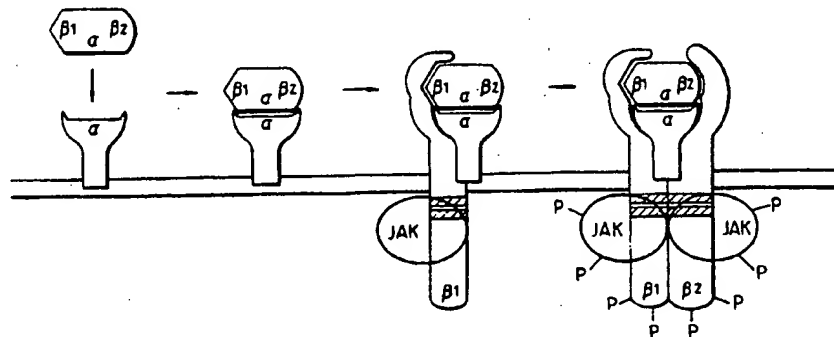


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<p>(21) International Application Number: PCT/US94/11873</p> <p>(22) International Filing Date: 19 October 1994 (19.10.94)</p> <p>(30) Priority Data: 08/140,222 20 October 1993 (20.10.93) US</p> <p>(71) Applicant: REGENERON PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US).</p> <p>(72) Inventors: STAHL, Neil; RD 10, Kent Shore Drive, Carmel, NY 10512 (US). ECONOMIDES, Aris; Apartment 11E, 150 West 96th Street, New York, NY 10025 (US). YAN-COPOULOS, George, D.; 1519 Baptist Church Road, Yorktown Heights, NY 10598 (US).</p> <p>(74) Agents: KEMPLER, Gail, M.; Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US) et al.</p>		<p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>

(54) Title: CNTF FAMILY ANTAGONISTS



## (57) Abstract

Heterodimer proteins comprising a soluble  $\alpha$  specificity determining cytokine receptor component and the extracellular domain of a  $\beta$  receptor component function as CNTF and IL-6 antagonists.

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## CNTF FAMILY ANTAGONISTS

### BACKGROUND OF THE INVENTION

Although discovered for varying biological activities, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and interleukin-6 (IL-6) comprise a newly defined family of cytokines (referred to herein as the "CNTF family" of cytokines). These cytokines are grouped together because of their distant structural similarities [Bazan, J. Neuron 7: 197-208 (1991); Rose and Bruce, Proc. Natl. Acad. Sci. USA 88: 8641-8645 (1991)], and, perhaps more importantly, because they share "B" signal-transducing receptor components [Baumann, et al., J. Biol. Chem. 265:19853-19862 (1993); Davis, et al., Science 260: 1805-1808 (1993); Gearing et al., Science 255:1434-1437 (1992); Ip et al., Cell 69: 1121-1132 (1992); Stahl, et al., J. Biol. Chem. 268: 7628-7631 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Receptor activation by this family of cytokines results from either homo- or heterodimerization of these B components [Davis, et al. Science 260: 1805-1808 (1993), Murakami, et al., Science 260: 1808-1810 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. IL-6 receptor activation requires homodimerization of gp130 [Murakami, et al. Science 260: 1808-1810 (1993), Hibi, et al., Cell 63: 1149-1157 (1990)], a protein initially identified as the IL-6 signal transducer [Hibi, et al., Cell 63: 1149-1157 (1990)]. CNTF, LIF and OSM receptor activation results from heterodimerization

between gp130 and a second gp130-related protein known as LIFR $\beta$  [Davis, et al., Science 260: 1805-1808 (1993)], that was initially identified by its ability to bind LIF [Gearing et al., EMBO J. 10: 2839-2848 (1991)].

5 In addition to the  $\beta$  components, some of these cytokines also require specificity-determining " $\alpha$ " components that are more limited in their tissue distribution than the  $\beta$  components, and thus determine the cellular targets of the particular cytokines [Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Thus,  
10 LIF and OSM are broadly acting factors that may only require the presence of gp130 and LIFR $\beta$  on responding cells, while CNTF requires CNTFR $\alpha$  [Stahl and Yancopoulos, Cell 74: 587-590 (1993)] and IL-6 requires IL-6R $\alpha$  [Kishimoto, et al., Science 258: 593-597 (1992)]. Both CNTFR $\alpha$  (Davis et al., Science 259:1736-1739  
15 (1993) and IL-6R $\alpha$  [Hibi, et al. Cell 63:1149-1157, Murakami et al., Science 260:1808-1810 (1990); Taga, et al., Cell 58:573-581 (1989)] can function as soluble proteins, consistent with the notion that they do not interact with intracellular signaling molecules but that they serve to help their ligands interact with  
20 the appropriate signal transducing  $\beta$  subunits [Stahl and Yancopoulos, Cell 74: 587-590 (1993)].

Additional evidence from other cytokine systems also supports the notion that dimerization provides a common mechanism by which all cytokine receptors initiate signal  
25 transduction. Growth hormone (GH) serves as perhaps the best example in this regard. Crystallographic studies have revealed that each GH molecule contains two distinct receptor binding sites, both of which are recognized by the same binding domain in

the receptor, allowing a single molecule of GH to engage two receptor molecules [de Vos, et al., Science 255: 306-312 (1992)]. Dimerization occurs sequentially, with site 1 on the GH first binding to one receptor molecule, followed by the binding of site 5 2 to a second receptor molecule [Fuh, et al., Science 256: 1677-1680 (1992)]. Studies with the erythropoietin (EPO) receptor are also consistent with the importance of dimerization in receptor activation, as EPO receptors can be constitutively activated by a single amino acid change that introduces a cysteine residue and 10 results in disulfide-linked homodimers [Watowich, et al., Proc. Natl. Acad. Sci. USA 89:2140-2144 (1992)].

In addition to homo- or hetero-dimerization of  $\beta$  subunits as the critical step for receptor activation, a second important feature is that formation of the final receptor complex by the 15 CNTF family of cytokines occurs through a mechanism whereby the ligand successively binds to receptor components in an ordered manner [Davis, et al. Science 260:1805-1818 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Thus CNTF first binds to CNTFR $\alpha$ , forming a complex which then binds gp130 to 20 form an intermediate (called here the  $\alpha\beta 1$  intermediate) that is not signaling competent because it has only a single  $\beta$  component, before finally recruiting LIFR $\beta$  to form a heterodimer of  $\beta$  components which then initiates signal transduction. Although a similar intermediate containing IL-6 bound to IL-6R $\alpha$  and a single 25 molecule of gp130 has not been directly isolated, we have postulated that it does exist by analogy to its distant relative, CNTF, as well as the fact that the final active IL-6 receptor complex recruits two gp130 monomers. Altogether, these

findings led to a proposal for the structure of a generic cytokine receptor complex (Figure 1) in which each cytokine can have up to 3 receptor binding sites: a site that binds to an optional  $\alpha$  specificity-determining component ( $\alpha$  site), a site that binds to the first  $\beta$  signal-transducing component ( $\beta 1$  site), and a site that binds to the second  $\beta$  signal-transducing component ( $\beta 2$  site) [Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. These 3 sites are used in sequential fashion, with the last step in complex formation -- resulting in  $\beta$  component dimerization -- critical for initiating signal transduction [Davis, et al. Science 260:1805-1818 (1993)]. Knowledge of the details of receptor activation and the existence of the non-functional  $\beta 1$  intermediate for CNTF has led to the finding that CNTF is a high affinity antagonist for IL-6 under certain circumstances, and provides the strategic basis for designing ligand or receptor-based antagonists for the CNTF family of cytokines as detailed below.

Once cytokine binding induces receptor complex formation, the dimerization of  $\beta$  components activates intracellular tyrosine kinase activity that results in phosphorylation of a wide variety of substrates [Ip, et al. Cell 69:121-1132 (1992)]. This activation of tyrosine kinase appears to be critical for downstream events since inhibitors that block the tyrosine phosphorylations also prevent later events such as gene inductions [Ip, et al., Cell 69:121-1132 (1992); Nakajima and Wall, Mol. Cell. Biol. 11:1409-1418 (1991)]. Recently, we have demonstrated that a newly discovered family of non-receptor tyrosine kinases that includes Jak1, Jak2, and Tyk2 (referred to as the Jak/Tyk kinases) [Firmbach-Kraft, et al., Oncogene 5:1329-

1336 (1990); Wilks, et al., Mol. Cell. Biol. 11: 2057-2065 (1991]  
and that are involved in signal transduction with other cytokines  
[, et al., Cell 74:237-244 (1993); Silvennoinen, et al., Proc. Natl.  
Acad. Sci. USA (in press; 1993); Velazquez, et al., Cell 70: 313-  
5 322 (1992); Witthuhn, et al., Cell 74:227-236 (1993)),  
preassociate with the cytoplasmic domains of the  $\beta$  subunits  
gp130 and LIFR $\beta$  in the absence of ligand, and become tyrosine  
phosphorylated and activated upon ligand addition [Stahl et al.,  
Science (submitted; 1993)]. Therefore these kinases appear to be  
10 the most proximal step of intracellular signal transduction  
activated inside the cell as a result of ligand binding outside of  
the cell. Assay systems for screening collections of small  
molecules for specific agonist or antagonist activities based on  
this system are described below.

15 The CNTF family of cytokines play important roles in a wide  
variety of physiological processes that provide potential  
therapeutic applications for both antagonists and agonists.

#### SUMMARY OF THE INVENTION

20 An object of the present invention is the production of IL-6  
antagonists that are useful in the treatment of IL-6 related  
diseases or disorders.

Another object of the invention is the use of IL-6  
antagonists described herein for the treatment of osteoporosis.

25 Another object of the invention is the use of IL-6  
antagonists described herein for the treatment of both the  
primary and second effects of cancers, including multiple  
myeloma.

Yet another object of the invention is the use of IL-6 antagonists described herein for the treatment of cachexia.

Another object of the invention is the development of screening systems useful for identifying novel agonists and antagonists of members of the CNTF family of cytokines.

Another object of the invention is the development of screening systems useful for identifying small molecules that act as agonists or antagonists of the CNTF family of cytokines.

These and other objects are achieved by the use of CNTF family receptor components to produce nonfunctional intermediates which have both therapeutic activity as IL-6 and CNTF antagonists, as well as utility in assay systems useful for identifying novel agonists and antagonists of the CNTF cytokine family members.

#### BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: Ordered binding of receptor components in a model of a generic cytokine receptor. The model indicates that cytokines contain up to 3 receptor binding sites and interact with their receptor components by binding first the optional  $\alpha$  component, followed by binding to  $\beta 1$ , and then  $\beta 2$ . The  $\beta$  components for many cytokine receptors interact through membrane proximal regions (shaded boxes) with the Jak/Tyk family of cytoplasmic protein tyrosine kinases. Only upon dimerization of  $\beta$  components is signal transduction initiated, as schematized by the tyrosine phosphorylations (P) of the  $\beta$  components and the Jak/Tyk kinases.



FIGURE 2: CNTF inhibits IL-6 responses in a PC12 cell line (called PC12D) that expresses IL6R $\alpha$ , gp130, CNTFR $\alpha$ , but not LIFR $\beta$ .

Serum-deprived PC12D cells were incubated + IL-6 (50 ng/mL) in  
5 the presence or absence of CNTF as indicated. Some plates also  
received soluble IL6R $\alpha$  (1 mg/mL) or soluble CNTFR $\alpha$  (1 mg/mL)  
as indicated. Cell lysates were subjected to immunoprecipitation  
with anti-gp130 and immunoblotted with anti-phosphotyrosine.  
Tyrosine phosphorylation of gp130 is indicative of IL-6 induced  
10 activation of the IL-6 receptor system, which is blocked upon  
coaddition of CNTF.

FIGURE 3: Scatchard analysis of iodinated CNTF binding on PC12D  
cells. PC12D cells were incubated with various concentrations of  
15 iodinated CNTF in the presence or absence of excess non-  
radioactive competitor to determine the specific binding. The  
figure shows a Scatchard plot of the amount of iodinated CNTF  
specifically bound, and gives data consistent with two binding  
sites with dissociation constants of 9 pM and 3.4 nM.

20

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel antagonists which are  
based on receptor components that are shared by cytokines such  
25 as the CNTF family of cytokines.

The invention described herein contemplates the production  
of antagonists to any cytokine that utilizes an  $\alpha$  specificity  
determining component which, when combined with the cytokine,

binds to a first  $\beta$  signal transducing component to form a nonfunctional intermediate which then binds to a second  $\beta$  signal transducing component causing  $\beta$ -receptor dimerization and consequent signal transduction. According to the invention, the soluble  $\alpha$  specificity determining component of the receptor ( $sR\alpha$ ) and the extracellular domain of the first  $\beta$  signal transducing component of the cytokine receptor ( $\beta 1$ ) are combined to form heterodimers ( $sR\alpha:\beta 1$ ) that act as antagonists to the cytokine by binding the cytokine to form a nonfunctional complex.

As described in Example 1, CNTF and IL-6 share the  $\beta 1$  receptor component gp130. The fact that CNTF forms an intermediate with CNTFR $\alpha$  and gp130 can be demonstrated (Example 1) in cells lacking LIFR $\beta$ , where the complex of CNTF and CNTFR $\alpha$  binds gp130, and prevents homodimerization of gp130 by IL-6 and IL-6R $\alpha$ , thereby blocking signal transduction. These studies provide the basis for the development of the IL-6 antagonists described herein, as they show that if, in the presence of a ligand, a nonfunctional intermediate complex, consisting of the ligand, its  $\alpha$  receptor component and its  $\beta 1$  receptor component, can be formed, it will effectively block the action of the ligand. Other cytokines may use other  $\beta 1$  receptor components, such as LIFR $\beta$ , which may also be used to produce antagonists according to the present invention.

Thus for example, in one embodiment of the invention, effective antagonists of IL-6 or CNTF consist of heterodimers of the extracellular domains of the  $\alpha$  specificity determining components of their receptors ( $sIL-6R\alpha$  and  $sCNTFR\alpha$  respectively) and the extracellular domain of gp130. The

resultant heterodimers, which are referred to hereinafter as sIL-6R $\alpha$ : $\beta$ 1 and sCNTFR $\alpha$ : $\beta$ 1 respectively, function as high-affinity traps for IL-6 or CNTF, respectively, thus rendering the cytokine inaccessible to form a signal transducing complex with the native  
5 membrane-bound forms of their receptors.

Although soluble ligand binding domains from the extracellular portion of receptors have proven to be somewhat effective as traps for their ligands and thus act as antagonists [Bargetzi, et al., Cancer Res. 53:4010-4013 (1993); , et al., Proc.  
10 Natl. Acad. Sci. USA 89: 8616-8620 (1992); Mohler, et al., J. Immunol. 151: 1548-1561 (1993); Narazaki, et al., Blood 82: 1120-1126 (1993)], the IL-6 and CNTF receptors are unusual in that the  $\alpha$  receptor components constitute ligand binding domains that, in concert with their ligands, function effectively in soluble  
15 form as receptor agonists [Davis, et al. Science 259:1736-1739 (1993); Taga, et al., Cell 58: 573-581 (1989)]. The sR $\alpha$ : $\beta$ 1 heterodimers prepared according to the present invention provide effective traps for their ligands, binding these ligands with affinities in the picomolar range (based on binding studies for  
20 CNTF to PC12D cells) without creating functional intermediates.

The  $\alpha$  and  $\beta$  receptor extracellular domains may be prepared using methods known to those skilled in the art. The CNTFR $\alpha$  receptor has been cloned, sequenced and expressed [Davis, et al. (1991) Science 253:59-63 which is incorporated by reference in  
25 its entirety herein]. The cloning of LIFR $\beta$  and gp130 are described in Gearing et al. in EMBO J. 10:2839-2848 (1991), Hibi, et al. Cell 63:1149-1157 (1990) and in published PCT application

WO 93/10151 published May 27, 1993, all of which are incorporated by reference in their entirety herein.

The receptor molecules useful for practicing the present invention may be prepared by cloning and expression in a  
5 prokaryotic or eukaryotic expression system. The recombinant receptor gene may be expressed and purified utilizing any number of methods. The gene encoding the factor may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

10 The recombinant factors may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be  
15 extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

20 The sR $\alpha$ : $\beta$  heterodimeric receptors may be engineered using known fusion regions, as described in published PCT application WO 93/10151 published May 27, 1993 entitled "Receptor for Oncostatin M and Leukemia Inhibitory Factor" which describes production of  $\beta$  receptor heterodimers, or they may be prepared by  
25 crosslinking of extracellular domains by chemical means. The domains utilized may consist of the entire extracellular domain of the  $\alpha$  and  $\beta$  components, or they may consist of mutants or

fragments thereof that maintain the ability to form a complex with its ligand and other components in the sR $\alpha$ : $\beta$ 1 complex.

In one embodiment of the invention, the extracellular domains are engineered using leucine zippers. The leucine zipper domains of the human transcription factors c-jun and c-fos have  
5 been shown to form stable heterodimers [Busch and Sassone-Corsi, Trends Genetics 6: 36-40 (1990); Gentz, et al., Science 243: 1695-1699 (1989)] with a 1:1 stoichiometry. Although jun-jun homodimers have also been shown to form, they are about  
10 1000-fold less stable than jun-fos heterodimers. Fos-fos homodimers have not been detected.

The leucine zipper domain of either c-jun or c-fos are fused in frame at the C-terminus of the soluble or extracellular domains of the above mentioned receptor components by  
15 genetically engineering chimeric genes. The fusions may be direct or they may employ a flexible linker domain, such as the hinge region of human IgG, or polypeptide linkers consisting of small amino acids such as glycine, serine, threonine or alanine, at various lengths and combinations. Additionally, the chimeric  
20 proteins may be tagged by His-His-His-His-His (His6), [SEQ. ID NO. 1] to allow rapid purification by metal-chelate chromatography, and/or by epitopes to which antibodies are available, to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

25 In another embodiment, the sR $\alpha$ : $\beta$ 1 heterodimer is prepared using a similar method, but using the Fc-domain of human IgG1 [Aruffo, et al., Cell 67:35-44 (1991)]. In contrast to the latter, formation of heterodimers must be biochemically achieved, as

chimeric molecules carrying the Fc-domain will be expressed as disulfide-linked homodimers. Thus, homodimers may be reduced under conditions that favor the disruption of inter-chain disulfides but do not effect intra-chain disulfides. Then

5 monomers with different extracellular portions are mixed in equimolar amounts and oxidized to form a mixture of homo- and heterodimers. The components of this mixture are separated by chromatographic techniques. Alternatively, the formation of this type of heterodimers may be biased by genetically engineering  
10 and expressing molecules that consist of the soluble or extracellular portion of the receptor components followed by the Fc-domain of hlgG, followed by either the c-jun or the c-fos leucine zippers described above [Kostelny, et al., J. Immunol. 148: 1547-1553 (1992)]. Since these leucine zippers form  
15 predominately heterodimers, they may be used to drive formation of the heterodimers where desired. As for the chimeric proteins described using leucine zippers, these may also be tagged with metal chelates or an epitope. This tagged domain can be used for rapid purification by metal-chelate chromatography, and/or by  
20 antibodies, to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

In another embodiment of the invention the sR $\alpha$ : $\beta$ 1 heterodimers are prepared by expression as chimeric molecules utilizing flexible linker loops. A DNA construct encoding the  
25 chimeric protein is designed such that it expresses two soluble or extracellular domains fused together in tandem ("head to head") by a flexible loop. This loop may be entirely artificial (e.g. polyglycine repeats interrupted by serine or threonine at a

- certain interval) or "borrowed" from naturally occurring proteins (e.g. the hinge region of hIgG). Molecules may be engineered in which the order of the soluble or extracellular domains fused is switched (e.g. sIL6R $\alpha$ /loop/sgp130 or sgp130/loop/sIL-6R $\alpha$ )
- 5 and/or in which the length and composition of the loop is varied, to allow for selection of molecules with desired characteristics.

Alternatively, the heterodimers made according to the present invention may be purified from cell lines cotransfected with the appropriate  $\alpha$  and  $\beta$  components. Heterodimers may be

10 separated from homodimers using methods available to those skilled in the art. For example, limited quantities of heterodimers may be recovered by passive elution from preparative, nondenaturing polyacrylamide gels. Alternatively, heterodimers may be purified using high pressure cation exchange

15 chromatography. Excellent purification has been obtained using a Mono S cation exchange column.

In addition to sR $\alpha$ : $\beta$ 1 heterodimers that act as antagonists by binding free CNTF or IL-6, the present invention also contemplates the use of engineered, mutated versions of IL-6

20 with novel properties that allow it to bind to IL-6R $\alpha$  and a single gp130 molecule, but fail to engage the second gp130 to complete  $\beta$  component homodimerization, and thus act as an effective IL-6 antagonist on any IL-6 responsive cell. Our model for the structure of the IL-6 and CNTF receptor complexes indicates that

25 these cytokines have distinct sites for binding the  $\alpha$ ,  $\beta$ 1, and  $\beta$ 2 receptor components [Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Mutations of critical amino acid residues comprising each of these sites gives rise to novel molecules which have the

desired antagonistic properties. Ablation of the  $\beta 1$  site would give a molecule which could still bind to the  $\alpha$  receptor component but not the  $\beta 1$  component, and thereby comprise an antagonist with nanomolar affinity. Mutations of critical amino acid residues comprising the  $\beta 2$  site of IL-6 (IL-6 $\beta 2^-$ ) would give a molecule that would bind to IL-6R $\alpha$  and the first gp130 monomer, but fail to engage the second gp130 and thus be functionally inactive. Similarly, mutations of the CNTF  $\beta 2$  site would give a molecule (CNTF $\beta 2^-$ ) that would bind CNTFR $\alpha$  and gp130, but fail to engage LIFR $\beta$ , thereby antagonizing CNTF action by forming the non-functional  $\beta 1$  intermediate. Based on the binding results described above where CNTF forms the  $\beta 1$  intermediate with high affinity, both CNTF $\beta 2^-$  and IL-6 $\beta 2^-$  would constitute antagonists with affinity in the range of 10 pM.

A variety of means are used to generate and identify mutations of IL-6 or CNTF that have the desired properties. Random mutagenesis by standard methods of the DNA encoding IL-6 or CNTF may be used, followed by analysis of the collection of products to identify mutated cytokines having the desired novel properties as outlined below. Mutagenesis by genetic engineering has been used extensively in order to elucidate the structural organization of functional domains of recombinant proteins. Several different approaches have been described in the literature for carrying out deletion or substitution mutagenesis. The most successful appear to be alanine scanning mutagenesis [Cunningham and Wells (1989), Science 244: 1081-1085] and homolog-scanning mutagenesis [Cunningham, et al., (1989), Science 243:1330-1336].



Targeted mutagenesis of the IL-6 or CNTF nucleic acid sequences using such methods can be used to generate CNTF $\beta$ 2- or IL-6 $\beta$ 2- candidates. The choice of regions appropriate for targeted mutagenesis is done systematically, or determined from studies whereby panels of monoclonal antibodies against each factor are used to map regions of the cytokine that might be exposed after binding of the cytokine to the  $\alpha$  receptor component alone, or to the  $\alpha\beta$ 1 heterodimeric soluble receptors described above. Similarly, chemical modification or limited proteolysis of the cytokine alone or in a complex bound to the  $\alpha$  receptor component or the  $\alpha\beta$ 1 heterodimeric soluble receptors described above, followed by analysis of the protected and exposed regions could reveal potential  $\beta$ 2 binding sites.

Assays for identifying CNTF or IL-6 mutants with the desired properties involve the ability to block with high affinity the action of IL-6 or CNTF on appropriately responsive cell lines [Davis, et al., Science 259: 1736-1739 (1993); Murakami et al., Proc. Natl. Acad. Sci. USA 88: 11349-11353 (1991)]. Such assays include cell proliferation, survival, or DNA synthesis driven by CNTF or IL-6, or the construction of cell lines where binding of factor induces production of reporters such as CAT or  $\beta$ -galactosidase [Savino, et al., Proc. Natl. Acad. Sci. USA 90: 4067-4071 (1993)].

Alternatively, the properties of various mutants may be assessed with a receptor-based assay. One such assay consists of screening mutants for their ability to bind the sR $\alpha$ : $\beta$ 1 receptor heterodimers described above using epitope-tagged [Davis et al., Science 253: 59-63 (1991)] sR $\alpha$ : $\beta$ 1 reagents. Furthermore, one

can probe for the presence or absence of the  $\beta 2$  site by assessing whether an epitope-tagged soluble  $\beta 2$  reagent will bind to the cytokine in the presence of the  $\alpha:\beta 1$  heterodimer. For example, CNTF only binds to LIFR $\beta$  (the  $\beta 2$  component) in the presence of both CNTFR $\alpha$  and gp130 [Davis, et al. Science 260: 1805-1808 (1993); Stahl, et al. J. Biol. Chem. 268: 7628-7631 (1993)]. Thus a soluble LIFR $\beta$  reagent would only bind to CNTF in the presence of the soluble sR $\alpha:\beta 1$  dimer sCNTFR $\alpha:\beta 1$ . For IL-6, the sR $\alpha:\beta 1$  reagent would be IL-6R $\alpha:\beta 1$ , and the probe for the  $\beta 2$  site would be epitope-tagged sgp130. Thus  $\beta 2^-$  mutants of CNTF would be identified as those that bound the sR $\alpha:\beta 1$  reagent, demonstrating that the  $\alpha$  and  $\beta 1$  site of the cytokine were intact, yet failed to bind the  $\beta 2$  reagent.

In addition, the present invention provides for methods of detecting or measuring the activity of potential  $\beta 2^-$  mutants by measuring the phosphorylation of a  $\beta$ -receptor component or a signal transduction component selected from the group consisting of Jak1, Jak2 and Tyk2 or any other signal transduction component, such as the CLIPs, that are determined to be phosphorylated in response to a member of the CNTF family of cytokines.

A cell that expresses the signal transduction component(s) described herein may either do so naturally or be genetically engineered to do so. For example, Jak1 and Tyk-2-encoding nucleic acid sequences obtained as described in Velazquez, et al., Cell, Vol. 70:313-322 (1992), may be introduced into a cell by transduction, transfection, microinjection, electroporation, via a transgenic animal, etc., using any known method known in the art.

According to the invention, cells are exposed to a potential antagonist and the tyrosine phosphorylation of either the  $\beta$ -component(s) or the signal transduction component(s) are compared to the tyrosine phosphorylation of the same component(s) in the absence of the potential antagonist.

In another embodiment of the invention, the tyrosine phosphorylation that results from contacting the above cells with the potential antagonist is compared to the tyrosine phosphorylation of the same cells exposed to the parental CNTF family member. In such assays, the cell must either express the extracellular receptor ( $\alpha$ -component) or the cells may be exposed to the test agent in the presence of the soluble receptor component. Thus, for example, in an assay system designed to identify agonists or antagonists of CNTF, the cell may express the  $\alpha$ -component CNTFR $\alpha$ , the  $\beta$ -components gp130 and LIFR $\beta$  and a signal transducing component such as Jak1. The cell is exposed to test agents, and the tyrosine phosphorylation of either the  $\beta$ -components or the signal transducing component is compared to the phosphorylation pattern produced in the presence of CNTF.

Alternatively, the tyrosine phosphorylation which results from exposure to a test agent is compared to the phosphorylation which occurs in the absence of the test agent. Alternatively, an assay system, for example, for IL-6 may involve exposing a cell that expresses the  $\beta$ -component gp130 and a signal transducing protein such as Jak1, Jak2 or Tyk2 to a test agent in conjunction with the soluble IL-6 receptor.

In another embodiment of the invention the above approaches are used to develop a method for screening for small

molecule antagonists that act at various steps in the process of ligand binding, receptor complex formation, and subsequent signal transduction. Molecules that potentially interfere with ligand-receptor interactions are screened by assessing interference of complex formation between the soluble receptors and ligand as described above. Alternatively, cell-based assays in which IL-6 or CNTF induce response of a reporter gene are screened against libraries of small molecules or natural products to identify potential antagonists. Those molecules showing antagonist activity are rescreened on cell-based assays responding to other factors (such as GMCSF or factors like Neurotrophin-3 that activate receptor tyrosine kinases) to evaluate their specificity against the CNTF/IL-6/OSM/LIF family of factors. Such cell-based screens are used to identify antagonists that inhibit any of numerous targets in the signal transduction process.

In one such assay system, the specific target for antagonists is the interaction of the Jak/Tyk family of kinases [Firmbach-Kraft, Oncogene 5: 1329-1336 (1990); Wilks, et al., Mol. Cell. Biol. 11:2057-2065 (1991)] with the receptor  $\beta$  subunits. As described above, LIFR $\beta$  and gp130 preassociate with members of the Jak/Tyk family of cytoplasmic protein tyrosine kinases, which become activated in response to ligand-induced  $\beta$  component dimerization (Stahl, et al. Science (submitted; 1993). Thus small molecules that could enter the cell cytoplasm and disrupt the interaction between the  $\beta$  component and the Jak/Tyk kinase could potentially block all subsequent intracellular signaling. Such activity could be screened with an in vitro scheme that assessed the ability of small molecules to block the

interaction between the relevant binding domains of purified  $\beta$  component and Jak/Tyk kinase. Alternatively, one could easily screen for molecules that could inhibit a yeast-based assay of  $\beta$  component binding to Jak/Tyk kinases using the two-hybrid  
5 interaction system [Chien, et al., Proc. Natl. Acad. Sci. 88: 9578-9582 (1991)]. In such a system, the interaction between two proteins ( $\beta$  component and Jak/Tyk kinase or relevant domains thereof in this example) induces production of a convenient marker such as  $\beta$ -galactosidase. Collections of small molecules  
10 are tested for their ability to disrupt the desired interaction without inhibiting the interaction between two control proteins. The advantage of this screen would be the requirement that the test compounds enter the cell before inhibiting the interaction between the  $\beta$  component and the Jak/Tyk kinase.

15 The CNTF family antagonists described herein either bind to, or compete with the cytokines CNTF and IL-6. Accordingly, they are useful for treating diseases or disorders mediated by CNTF or IL-6. For example, therapeutic uses of IL-6 antagonists would include the following:

- 20 1) In osteoporosis, which can be exacerbated by lowering of estrogen levels in post-menopausal women or through ovariectomy, IL-6 appears to be a critical mediator of osteoclastogenesis, leading to bone resorption [Horowitz, Science 260: 626-627 (1993); Jilka, et al., Science 257: 88-91 (1992)].
- 25 Importantly, IL-6 only appears to play a major role in the estrogen-depleted state, and apparently is minimally involved in normal bone maintenance. Consistent with this, experimental evidence indicates that function-blocking antibodies to IL-6 can

reduce the number of osteoclasts [Jilka, et al. Science 257: 88-91 (1992)]. While estrogen replacement therapy is also used, there appear to be side effects that may include increased risk of endometrial and breast cancer. Thus, IL-6 antagonists as  
5 described herein would be more specific to reduce osteoclastogenesis to normal levels.

2) IL-6 appears to be directly involved in multiple myeloma by acting in either an autocrine or paracrine fashion to promote tumor formation [van Oers, et al., Ann Hematol. 66: 219-223  
10 (1993)]. Furthermore, the elevated IL-6 levels create undesirable secondary effects such as bone resorption, hypercalcemia, and cachexia; in limited studies function-blocking antibodies to IL-6 or IL-6R $\alpha$  have some efficacy [Klein, et al., Blood 78: 1198-1204 (1991); Suzuki, et al., Eur. J. Immunol. 22:1989-1993 (1992)].  
15 Therefore, IL-6 antagonists as described herein would be beneficial for both the secondary effects as well as for inhibiting tumor growth.

3) IL-6 may be a mediator of tumor necrosis factor (TNF) that leads to cachexia associated with AIDS and cancer  
20 [Strassmann, et al., J. Clin. Invest. 89: 1681-1684 (1992)], perhaps by reducing lipoprotein lipase activity in adipose tissue [Greenberg, et al., Cancer Research 52: 4113-4116 (1992)]. Accordingly, antagonists described herein would be useful in alleviating or reducing cachexia in such patients.

25 Effective doses useful for treating these or other CNTF family related diseases or disorders may be determined using methods known to one skilled in the art [see, for example, Fingl, et al., The Pharmacological Basis of Therapeutics, Goodman and

Gilman, eds. Macmillan Publishing Co., New York, pp. 1-46

((1975)). Pharmaceutical compositions for use according to the invention include the antagonists described above in a pharmacologically acceptable liquid, solid or semi-solid carrier, linked to a carrier or targeting molecule (e.g., antibody, hormone, growth factor, etc.) and/or incorporated into liposomes, microcapsules, and controlled release preparation (including antagonist expressing cells) prior to administration in vivo. For example, the pharmaceutical composition may comprise one or more of the antagonists in an aqueous solution, such as sterile water, saline, phosphate buffer or dextrose solution.

Alternatively, the active agents may be comprised in a solid (e.g. wax) or semi-solid (e.g. gelatinous) formulation that may be implanted into a patient in need of such treatment. The

administration route may be any mode of administration known in the art, including but not limited to intravenously, intrathecally, subcutaneously, by injection into involved tissue, intraarterially, intranasally, orally, or via an implanted device.

Administration may result in the distribution of the active agent of the invention throughout the body or in a localized area. For example, in some conditions which involve distant regions of the nervous system, intravenous or intrathecal administration of agent may be desirable. In some situations, an implant containing active agent may be placed in or near the lesioned area. Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

EXAMPLE 1: CNTF COMPETES WITH IL-6 FOR BINDING TO  
GP130

MATERIALS AND METHODS

Materials. A clone of PC12 cells that respond to IL-6  
5 (PC12D) was obtained from DNAX. Rat CNTF was prepared as  
described [Masiakowski, et al., J. Neurochem. 57:1003-10012  
(1991)]. IL-6 and sIL-6R were purchased from R & D Systems.  
Antisera was raised in rabbits against a peptide derived from a  
region near the C-terminus of gp130 (sequence:  
10 CGTEGQVERFETVGME) [SEQ. ID. NO. 2] by the method described  
(Stahl, et al. J. Biol. Chem. 268:7628-7631 (1993)). Anti-  
phosphotyrosine monoclonal 4G10 was purchased from UBI, and  
reagents for ECL from Amersham.

Signal Transduction Assays. Plates (10 cm) of PC12D were  
15 starved in serum-free medium (RPMI 1640 + glutamine) for 1  
hour, then incubated with IL-6 (50 ng/mL) + sIL-6R (1 µg/mL) in  
the presence or absence of added rat CNTF at the indicated  
concentrations for 5 minutes at 37°C. Samples were then  
subjected to anti-gp130 immunoprecipitation, SDS PAGE, and  
20 anti-phosphotyrosine immunoblotting as described (Stahl, et al. J.  
Biol. Chem. 268:7628-7631 (1993)).

RESULTS

The ability of CNTF to block IL-6 responses was measured  
using a PC12 cell line (called PC12D) that expresses IL-6R $\alpha$ ,  
25 gp130, and CNTFR $\alpha$ , but not LIFR $\beta$ . As one would predict, these  
cells respond to IL-6, but not to CNTF (Fig. 2) since LIFR $\beta$  is a  
required component for CNTF signal transduction [Davis, et al.,  
Science 260: 59-63 (1993)]. In accordance with results on other



cell lines [Ip, et al., Cell 69: 1121-1132 (1992)], PC12D cells give tyrosine phosphorylation of gp130 (as well as a variety of other proteins called CLIPs) in response to 2 nM IL-6 (Fig. 2). Addition of recombinant soluble IL-6R $\alpha$  (sIL-6R $\alpha$ ) enhances the level of gp130 tyrosine phosphorylation, as has been reported in some other systems [(Taga, et al., Cell 58: 573-581 (1989)]. However, addition of 2 nM CNTF simultaneously with IL-6 severely diminishes the tyrosine phosphorylation of gp130. Although a slight gp130 phosphorylation response remains in the presence of CNTF, IL-6, and sIL-6R $\alpha$ , it is eliminated if the CNTF concentration is increased fourfold to 8 nM. Thus, in IL-6 responsive cells that contain CNTFR $\alpha$  but no LIFR $\beta$ , CNTF is a rather potent antagonist of IL-6 action.

## EXAMPLE 2. BINDING OF CNTF TO THE CNTFR $\alpha$ : $\beta$

### MATERIALS AND METHODS

Scatchard Analysis of CNTF Binding.  $^{125}$ I-CNTF was prepared and purified as described [Stahl et al. JBC 268: 7628-7631 (1993)]. Saturation binding studies were carried out in PC12 cells, using concentrations of  $^{125}$ I-CNTF ranging from 20pM to 10nM. Binding was performed directly on a monolayer of cells. Medium was removed from wells and cells were washed once with assay buffer consisting of phosphate buffered saline (PBS; pH 7.4), 0.1mM bacitracin, 1mM PMSF, 1 $\mu$ g/ml leupeptin, and 1mg/ml BSA. Cells were incubated in  $^{125}$ I-CNTF for 2 hours at room temperature, followed by 2 quick washes with assay buffer. Cells were lysed with PBS containing 1% SDS and counted in a

Packard Gamma Counter at 90-95% efficiency. Non-specific binding was defined by the presence of 100-fold excess of unlabelled CNTF. Specific binding ranged from 70% to 95%.

### RESULTS

5        The equilibrium constant for binding of CNTF to CNTFR $\alpha$ : $\beta$ 1 was estimated from Scatchard analysis of iodinated CNTF binding on PC12D cells (Figure 3). The data is consistent with a 2 site fit having dissociation constants of 9 pM and 3.4 nM. The low affinity site corresponds to interaction of CNTF with CNTFR $\alpha$  ,  
10        which has a Kd near 3 nM [(Panayotatos, et al., J. Biol. Chem. 268: 19000-19003 (1993)]. We interpret the high affinity complex as the intermediate containing CNTF, CNTFR $\alpha$  , and gp130. A Ewing sarcoma cell line (EW-1) which does contain CNTFR $\alpha$ , gp130, and LIFR $\beta$ , and therefore gives robust tyrosine phosphorylation in  
15        response to CNTF, displays a very similar two site fit with dissociation constants of 1 nM and 10 pM (Wong, et al., unpublished data). Thus it is apparent that CNTF binds with equally high affinity to a complex containing only CNTFR $\alpha$  and gp130, as it does to a complex which additionally contains LIFR $\beta$ ,  
20        thus demonstrating the feasibility of creating the sR $\alpha$ : $\beta$  antagonists described herein.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5 (i) APPLICANT: REGENERON PHARMACEUTICALS, INC.

(ii) TITLE OF INVENTION: CNTF Family Antagonists

10 (iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Regeneron Pharmaceuticals, Inc.

(B) STREET: 777 Old Saw Mill River Road

(C) CITY: Tarrytown

15 (D) STATE: New York

(E) COUNTRY: U.S.A.

(F) ZIP: 10591

(v) COMPUTER READABLE FORM:

20 (A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

25 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

30 (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kempler Ph.D., Gall M.

(B) REGISTRATION NUMBER: 32,143

(C) REFERENCE/DOCKET NUMBER: REG 200

35 (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 914-345-7400

(B) TELEFAX: 914-345-7721

40

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 6 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15 His His His His His His  
1 5

## (2) INFORMATION FOR SEQ ID NO:2:

## 20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

25

## (ii) MOLECULE TYPE: peptide

## 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys Gly Thr Glu Gly Gln Val Glu Arg Phe Glu Thr Val Gly Met Glu  
1 5 10 15

**CLAIMS**

1. A sR $\alpha$ : $\beta$ 1 heterodimeric protein comprising a soluble  $\alpha$  specificity determining component of a cytokine receptor and the soluble  $\beta$ 1 component of the cytokine receptor.
2. The protein of claim 1 wherein the cytokine is a member of the CNTF family of cytokines.
3. The protein of claim 1 wherein said  $\beta$ 1 receptor is gp130.
4. The protein of claim 1 wherein said  $\beta$ 1 receptor is LIFR $\beta$ .
5. The protein of claim 2 wherein the cytokine is CNTF, said  $\alpha$  receptor is sCNTFR $\alpha$  and said  $\beta$ 1 receptor is gp130.
6. The protein of claim 2 wherein the cytokine is IL-6, said  $\alpha$  receptor is sIL-6R $\alpha$  and said  $\beta$ 1 receptor is gp130.
7. An IL-6 protein comprising a  $\beta$ 2- mutation wherein said mutation renders said IL-6 incapable of binding a second gp130 molecule when said IL-6 is complexed with IL-6R $\alpha$  and a first gp130 molecule.

8. A CNTF protein comprising a  $\beta 2$ -mutation wherein said mutation renders said CNTF incapable of binding LIFR $\beta$  when said CNTF is complexed with CNTFR $\alpha$  and gp130.
9. A method of isolating IL-6 antagonists comprising
  - 1) obtaining an IL-6 producing clone
  - 2) mutating such clone using random or targeted mutagenesis to produce mutant IL-6 expressing subclones;
  - 3) recovering the mutant IL-6 molecules produced by the subclones;
  - 4) identifying as IL-6 antagonists the mutated IL-6 molecules incapable of binding a second gp130 when complexed with IL-6R $\alpha$  and a first gp130 molecule.
10. The method according to claim 9 wherein said identification comprises:
  - i) adding to the mutated IL-6 molecule IL-6R $\alpha$ : $\beta 1$  heterodimer to form an IL-6/IL-6R $\alpha$ : $\beta 1$  intermediate;
  - ii) adding to said intermediate detectable gp130 to form an IL-6:IL-6R $\alpha$ : $\beta 1$ : $\beta 2$  complex;
  - iii) determining the binding of said detectable gp130 to said IL-6/ $\alpha\beta 1$  intermediate by measuring the amount of the detectable gp130 in the IL-6:IL-6R $\alpha$ : $\beta 1$ : $\beta 2$  complex.
11. A method of isolating IL-6 antagonists comprising
  - 1) obtaining an IL-6 producing clone
  - 2) mutating such clone using random or targeted

- mutagenesis to produce mutant IL-6 expressing subclones;
- 3) expressing the mutated IL-6 molecules;
  - 4) combining said mutated IL-6 molecules with IL-6R $\alpha$  to form IL-6/IL-6R $\alpha$  complex;
  - 5) adding to said IL-6/IL-6R $\alpha$  complex detectable gp130;
  - 6) identifying as IL-6 antagonists said mutated IL-6 molecules incapable of binding said detectable gp130 when present in said IL-6/IL-6R $\alpha$  complex.
12. A method of isolating CNTF antagonists comprising
- 1) obtaining a CNTF producing clone;
  - 2) mutating such clone using random or targeted mutagenesis to produce mutant CNTF expressing subclones;
  - 3) recovering the mutant CNTF molecules produced by the subclones;
  - 4) identifying as CNTF antagonists the mutated CNTF molecules incapable of binding LIFR $\beta$  when complexed with CNTFR $\alpha$  and gp130.
13. The method according to claim 12 wherein said identifying comprises:
- i) adding to the mutated CNTF molecule CNTFR $\alpha$ : $\beta$ 1 heterodimer to form a CNTF/CNTFR $\alpha$ : $\beta$ 1 intermediate;
  - ii) adding to said intermediate detectable LIFR $\beta$  to form a CNTF:CNTFR $\alpha$ : $\beta$ 1: $\beta$ 2 complex;
  - iii) determining the binding of said detectable LIFR $\beta$  to said

CNTF/ $\alpha\beta 1$  intermediate by measuring the amount of detectable LIFR $\beta$  in the CNTF:CNTFR $\alpha$ : $\beta 1$ : $\beta 2$  complex.

14. A method of isolating CNTF antagonists comprising
  - 1) obtaining a CNTF producing clone
  - 2) mutating such clone using random or targeted mutagenesis to produce mutant CNTF expressing subclones;
  - 3) expressing the mutated CNTF molecules;
  - 4) combining said mutated CNTF molecules with CNTFR $\alpha$  to form CNTF/CNTFR $\alpha$  complex;
  - 5) adding to said CNTF/CNTFR $\alpha$  complex detectable gp130;
  - 6) identifying as CNTF antagonists CNTF molecules incapable of binding said detectable gp130 when present in said CNTF/CNTFR $\alpha$  complex.
15. A DNA sequence encoding a protein according to any one of claims 1-8.
16. A protein according to any one of claims 1, 7 or 8 hereinbefore specifically mentioned.
17. A pharmaceutical composition comprising a protein according to any one of claims 1 to 8 or 16 together with a pharmaceutically acceptable carrier.



18. A pharmaceutical composition for treating an IL-6 related disease or disorder comprising the protein of claim 6 or 7 and a pharmaceutically acceptable carrier.
19. A protein according to claim 6 or 7 for use in a method of treating osteoporosis in an estrogen depleted patient.
20. A protein according to claim 6 or 7 for use in a method of treating multiple myeloma in a patient.
21. A protein according to claim 6 or 7 for use in a method of treating cachexia in a patient.

Fig.1.

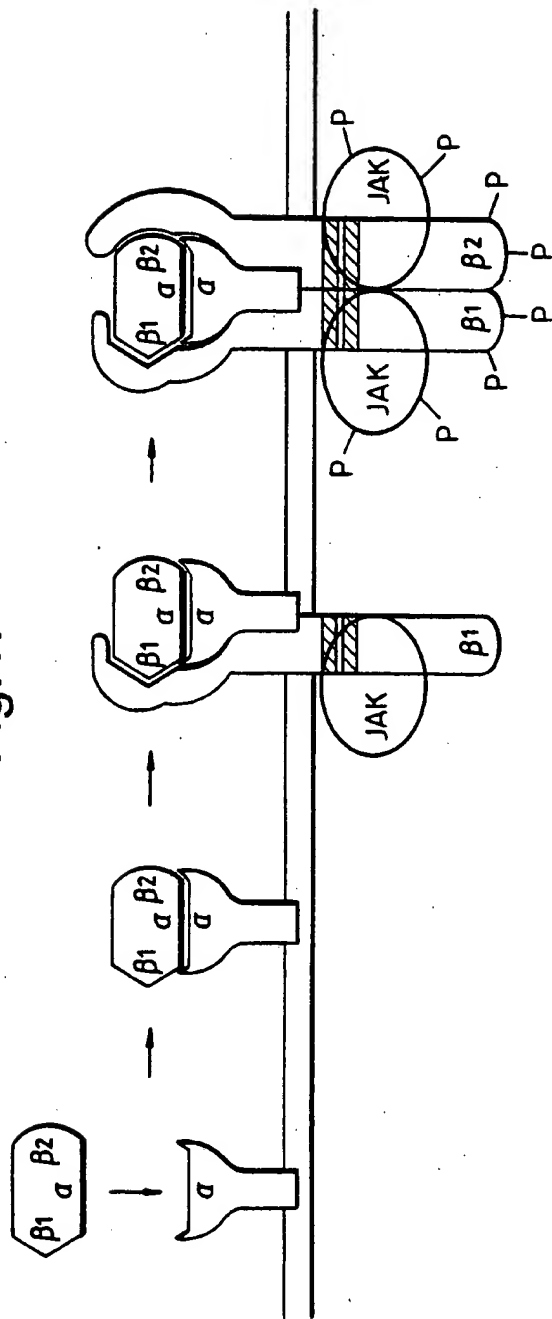


Fig.2.

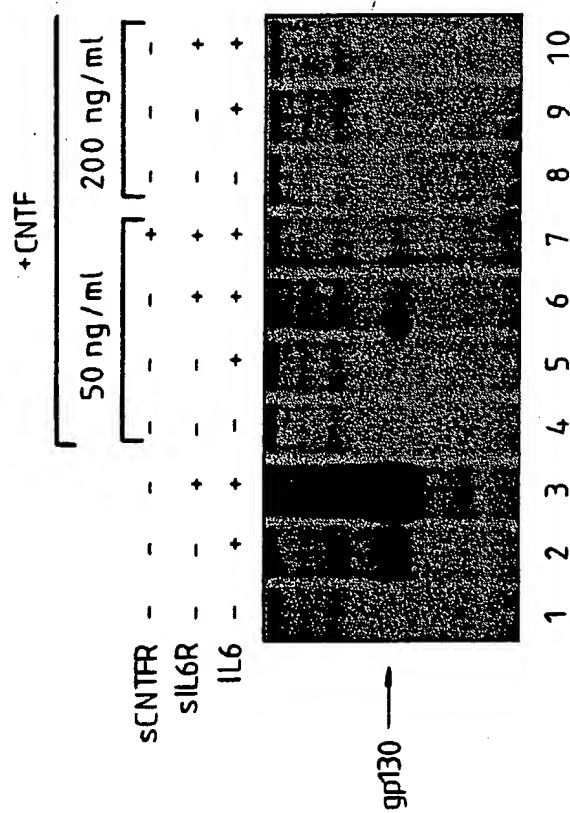
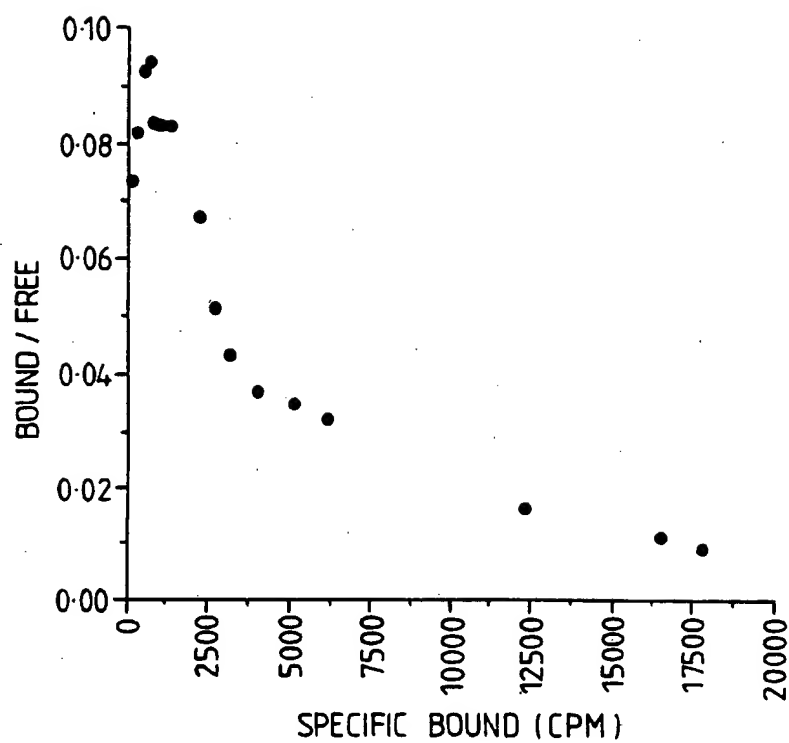


Fig.3.



## INTERNATIONAL SEARCH REPORT

Internat. Application No

PCT/US 94/11873

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/24 C07K14/54 C07K14/52 C07K14/715 C12N15/19  
 C12P21/02 A61K38/17 A61K38/20 A61K38/19

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CYTOKINE, vol.3, no.5, September 1991 page 496 J.P.J. BRAKENHOFF ET AL 'Construction of an interleukin-6 antagonist' * see abstract 279 *	11
A	WO,A,93 10151 (IMMUNEX CORPORATION) 27 May 1993 cited in the application see page 7, line 27 - page 9, line 37	1
A	CELL, vol.74, 27 August 1993, CAMBRIDGE, MA US pages 587 - 590 N. STAHL ET AL 'The alphas, betas, and kinases of cytokine receptor complexes' cited in the application	

-/--

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*A\* document member of the same patent family

Date of the actual completion of the international search

13 February 1995

Date of mailing of the international search report

22-02-1995

Name and mailing address of the ISA

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BLOOD, vol.82, no.4, 15 August 1993 pages 1120 - 1126 MASASHI NARAZAKI ET AL 'Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130' cited in the application * see the whole document especially page 1120 *</p> <p>---</p>	1-6
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol.268, no.11, 15 April 1993, BALTIMORE, MD US pages 7628 - 7631 N. STAHL ET AL 'Cross-linking identifies Leukemia inhibitory factor-binding protein as a Ciliary Neurotrophic Factor Receptor component' see the whole document</p> <p>---</p>	1-6
A	<p>IMMUNOLOGY LETTERS, vol.31, no.2, 1 February 1992 pages 123 - 130 K. YASUKAWA ET AL 'Association of recombinant soluble IL-6-signal transducer , gp130, with a complex of IL-6 and soluble IL-6 receptor , and establishment of an ELISA for soluble gp130' see page 126 see figure 3</p> <p>---</p>	1-3,6
A	<p>CURRENT OPINION IN CELL BIOLOGY, vol.5, no.2, April 1993 pages 281 - 285 S.DAVIS ET AL 'The molecular biology of the CNTF receptor' see the whole document</p> <p>---</p>	1-6
P,X	<p>EMBO JOURNAL, vol.13, no.6, 15 March 1994, EYNSHAM, OXFORD GB pages 1357 - 1367 R. SAVINO ET AL 'Generation of interleukin-6 receptor antagonists by molecular-modeling guided mutagenesis of residues important for gp130 activation' see the whole document</p> <p>---</p>	11

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### Information on patient family members

**PCT/US 94/11873**

Form PCT/ISA/218 (patent family annex) (July 1992)